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Fusarium and *Candida albicans* Biofilms on Soft Contact Lenses: Model Development, Influence of Lens Type, and Susceptibility to Lens Care Solutions[▽]

Yoshifumi Imamura,[1] Jyotsna Chandra,[1] Pranab K. Mukherjee,[1] Ali Abdul Lattif,[1] Loretta B. Szczotka-Flynn,[2] Eric Pearlman,[2] Jonathan H. Lass,[2] Kerry O'Donnell,[3] and Mahmoud A. Ghannoum[1]*

Center for Medical Mycology and Department of Dermatology[1] and Department of Ophthalmology and Visual Sciences,[2] University Hospitals Case Medical Center and Case Western Reserve University, Cleveland, Ohio, and National Center for Agricultural Utilization Research, ARS-USDA, Peoria, Illinois[3]

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Fungal keratitis is commonly caused by *Fusarium* species and less commonly by *Candida* species. Recent outbreaks of *Fusarium* keratitis were associated with contact lens wear and with ReNu with MoistureLoc contact lens care solution, and biofilm formation on contact lens/lens cases was proposed to play a role in this outbreak. However, no in vitro model for contact lens-associated fungal biofilm has been developed. In this study, we developed and characterized in vitro models of biofilm formation on various soft contact lenses using three species of *Fusarium* and *Candida albicans*. The contact lenses tested were etafilcon A, galyfilcon A, lotrafilcon A, balafilcon A, alphafilcon A, and polymacon. Our results showed that clinical isolates of *Fusarium* and *C. albicans* formed biofilms on all types of lenses tested and that the biofilm architecture varied with the lens type. Moreover, differences in hyphal content and architecture were found between the biofilms formed by these fungi. We also found that two recently isolated keratitis-associated fusaria formed robust biofilms, while the reference ATCC 36031 strain (recommended by the International Organization for Standardization guidelines for testing of disinfectants) failed to form biofilm. Furthermore, using the developed in vitro biofilm model, we showed that phylogenetically diverse planktonic fusaria and *Candida* were susceptible to MoistureLoc and MultiPlus. However, *Fusarium* biofilms exhibited reduced susceptibility against these solutions in a species- and time-dependent manner. This in vitro model should provide a better understanding of the biology and pathogenesis of lens-related fungal keratitis.

Fusarium keratitis is a devastating ocular disease and an important cause of morbidity and blindness (34, 47). This disease has been reported in different parts of the world, particularly in tropical areas (1, 2), where it may account for more than 50% of all ocular mycoses (3). A major increase in the incidence of fungal keratitis during the past 2 years has made it a relatively new public health concern, drawing broad international attention. Fungal keratitis is caused by filamentous fungi (especially *Fusarium* and *Aspergillus*) and by yeast-like fungi (particularly *Candida albicans*) (5).

Recently several investigations, including those by the Singapore Health Ministry and the U.S. Centers for Disease Control and Prevention (CDC) and in the French West Indies, reported outbreaks of *Fusarium* keratitis associated with contact lens wear (16, 18, 29). Although fungal keratitis is generally associated with trauma and prior application of corticosteroids (6), patients in these outbreaks had no history of recent ocular trauma (16). The majority of the confirmed *Fusarium* keratitis cases reported by the CDC indicated the wearing of soft contact lenses and use of the alexidine-containing contact lens cleaning solution ReNu with MoistureLoc ("MoistureLoc") (16). Also, as noted by the CDC, although the market share of ReNu Multipurpose solution ("MultiPlus," containing polyhexamethylene biguanide) was five times higher than that of MoistureLoc, the majority of reported *Fusarium* keratitis cases involved the use of the latter lens cleaning solution, thus implicating it in the outbreak (16).

C. albicans also causes fungal keratitis and lens-associated infections, although they are less common than *Fusarium*-associated keratitis. However, ocular infections caused by the former are usually associated with an underlying condition (25, 47). In a retrospective review of fungal keratitis cases at the University of Florida from 1999 to 2006, Iyer et al. (25) showed that the most commonly isolated fungi in these cases were *Fusarium* (41%) and *Candida* (14%), followed by *Curvularia* (12%) and *Aspergillus* (12%). Although contact lens-related infections associated with fungi, especially *Fusarium*, have gained prominence recently, Iyer et al. (25) showed that the incidence of this disease was increasing even before the latest outbreak. In this regard, before 2004, trauma was the most common risk factor for fungal keratitis (51%), compared to contact lens use (40%), while after 2005, this disease was associated more with contact lens use (risk factor 52%) than with trauma (29%). These studies underscore the need for detailed investigations into the biology and pathogenesis of *Fusarium* and non-*Fusarium* keratitis.

One mechanism by which keratitis-associated *Fusarium* ex-

* Corresponding author. Mailing address: Center for Medical Mycology, University Hospitals of Cleveland and Case Western Reserve University, 11100 Euclid Avenue, Cleveland, OH 44106. Phone: (216) 844-8580. Fax: (216) 844-1076. E-mail: mahmoud.ghannoum@case.edu.

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TABLE 1. Properties of soft contact lenses used in this study

Proprietary name (manufacturer)	Polymer type	Water content (%)	Ionic charge	FDA group
Acuvue (Vistakon)	Etafilcon A	58	Ionic	IV
Acuvue Advance (Vistakon)	Galyfilcon A	47	Nonionic	I (Silicone hydrogel, uncoated)
Night & Day (CIBA Vision)	Lotrafilcon A	24	Nonionic	I (Silicone hydrogel, plasma surface coating)
PureVision (Bausch & Lomb)	Balafilcon A	36	Ionic	III (Silicone hydrogel, plasma oxidation surface)
SoftLens66 (Bausch & Lomb)	Alphafilcon A	64	Nonionic	II
Nike Maxsight (Bausch & Lomb)	Polymacon	38	Nonionic	I

hibits a drug-resistant phenotype may be formation of biofilms (microbial communities that are embedded in an endogenous extracellular matrix). In this regard, bacterial biofilms have been demonstrated on contact lenses obtained from patients with culture-proven keratitis (20, 45, 50). However, investigations of fungal biofilms associated with contact lenses and lens cases are virtually nonexistent, and the lack of robust in vitro models has resulted in scant information about the pathogenesis of *Fusarium* keratitis.

In the current study, we established an in vitro model of contact lens-associated *Fusarium* and *Candida* biofilms and used this model to characterize biofilms formed by these organisms on soft contact lenses belonging to the four FDA-designated groups (groups I, II, III, and IV). In addition, silicone hydrogel lenses, which represent a lens type with different monomer polymer backbones and surface treatments and greatly enhanced oxygen permeability compared to traditional hydrogels (10), were also examined. Finally, we used our in vitro model to determine whether biofilms formed by *Fusarium* and *Candida* isolates were susceptible to MoistureLoc and MultiPlus contact lens care solutions. Our results showed that both *Fusarium* and *C. albicans* can form biofilms on the different lens types tested, but there were pronounced differences in biofilm architecture, hyphal morphology, biofilm thickness, and metabolic activity between *Fusarium* and *Candida*. Furthermore, while the two contact lens solutions tested were active against the planktonic (free-floating) forms of *Fusarium* and *Candida*, these solutions were less effective against *Fusarium* biofilms in a strain- and time-dependent manner. Moreover, these solutions were ineffective against *Candida* biofilms. Our studies suggest that the suboptimal activity of MoistureLoc against *Fusarium* infections in recent outbreaks may be due to the ability of these pathogens to form resistant biofilms.

MATERIALS AND METHODS

Fungal strains. The three keratitis-associated fusaria included in this study were characterized using DNA sequence data from three loci as previously described (16). Results of multilocus DNA sequence typing indicate that the fusaria represent three phylogenetically distinct species. Two of these are members of the species-rich *Fusarium solani* species complex (FSSC)] (51), while the third is a member of the *Fusarium oxysporum* species complex (FOSC) 3-a widespread clonal lineage (40). FSSC multilocus haplotype 1-b strain MRL8609 (= NRRL 47513) was isolated from a patient with fungal keratitis not associated with contact lens use at University Hospitals Case Medical Center, Cleveland, OH, while the FOSC 3-a strain MRL8996 (= NRRL 47514) was isolated from a patient with contact lens-associated fungal keratitis at Cleveland Clinic Foundation. ATCC 36031 (= NRRL 47512) FSSC multilocus haplotype 2-c, which was originally isolated from a human corneal ulcer in Nigeria in the mid-1970s, was acquired from the American Type Culture Collection (Manassas, VA) and used as a reference isolate in our *Fusarium*-related experiments. The ATCC 36031 strain is the recommended reference isolate in the International Organization for Standardization (ISO 14729) guidelines for testing of the antimicrobial activity of lens care solutions in vitro. *C. albicans* strain SC5314, a clinical isolate obtained from a candidiasis patient, was a generous gift from William Fonzi (Georgetown University, Washington, DC). All of the fusaria are available upon request from the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research, Peoria, IL.

Fungal growth conditions. *Fusarium* isolates were grown at 37°C for 40 h in Sabouraud dextrose broth (SDB) (Difco Laboratories, Detroit, MI). To collect conidia following incubation, conidia of the *Fusarium* species were harvested and hyphae were removed by filtration through sterile gauze. Conidia were washed with phosphate-buffered saline (PBS) and standardized to 1×10^3 conidia/ml for growth rate studies and 1×10^6 conidia/ml for biofilm formation experiments. *C. albicans* was grown overnight at 37°C in yeast nitrogen base medium (YNB) (Difco Laboratories) supplemented with 50 mM glucose. *Candida* cells were harvested, washed with PBS, and standardized to 1×10^7 blastospores/ml for biofilm formation experiments. Growth of *Fusarium* in SDB, YNB, yeast potato dextrose (YPD) broth (Difco Laboratories), and RPMI 1640 (Mediatech, Inc., Herndon, VA) was monitored to identify the optimal medium to be used for biofilm formation by fusaria.

Soft contact lenses and lens care solutions. Soft contact lenses used in the present study included the following: etafilcon A (Vistakon; Johnson & Johnson, Jacksonville, FL), galyfilcon A (Vistakon), lotrafilcon A (CIBA Vision, Duluth, GA), balafilcon A (Bausch & Lomb, Rochester, NY), alphafilcon A (Bausch & Lomb), and polymacon (Bausch & Lomb). Properties (water content and surface charge) of these contact lenses are shown in Table 1. All the lenses used had a power of +1.50 diopters. The lens care solutions tested were MoistureLoc (lot number GG5033; Bausch & Lomb) and MultiPlus (lot number GF6002; Bausch & Lomb). Lens care solutions were stored at room temperature as per the manufacturer's suggestions.

Biofilm formation. To evaluate biofilm formation by *Fusarium* and *Candida* isolates, soft contact lenses were washed with PBS, placed in 12-well tissue culture plates with 4 ml standardized cell suspension (1×10^7 cells/ml for *C. albicans* and 1×10^6 conidia/ml for the fusaria), and incubated for 90 min at 37°C (adherence phase). Nonadherent cells were removed from soft contact lenses by gentle washing with 4 ml PBS. Next, soft contact lenses were immersed in medium (SDB for the fusaria, and YNB for *C. albicans*) and incubated for 48 h at 37°C on a rocker. Biofilms were quantified using a tetrazolium XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay as described previously (13). Planktonic cells were grown in 12-well plates in the absence of a contact lens.

Gross morphology of biofilms formed on contact lenses. To characterize the gross morphology of biofilms formed on contact lenses, lenses were seeded with fungi and allowed to adhere and then form biofilms for 48 h. Next, a digital camera was used to capture images of the biofilms to compare their gross morphologies and appearances.

CSLM. The architecture of biofilms formed on soft contact lenses was analyzed using confocal scanning laser microscopy (CSLM), following our previously described method (13). Briefly, soft contact lenses containing biofilms were transferred to 12-well plates and incubated for 45 min at 37°C in 4 ml of PBS containing the fluorescent stains FUN-1 (10 mM) and concanavalin A-Alexa Fluor 488 conjugate (ConA) (25 mg/ml). FUN-1 (long-pass filter; excitation wavelength, 543 nm; emission, 560 nm) is converted to orange-red cylindrical intravacuolar structures by metabolically active cells, while ConA (long-pass filter; excitation wavelength, 488 nm; emission, 505 nm) binds to glucose and mannose residues of fungal cell wall polysaccharides and emits green fluorescence. After incubation with the dyes, the lenses were flipped and placed on a 35-mm-diameter glass-bottom petri dish (MatTek Corp., Ashland, MA). Stained biofilms were observed by using a Zeiss LSM510 confocal scanning laser microscope equipped with argon and HeNe lasers and mounted on a Zeiss Axio-

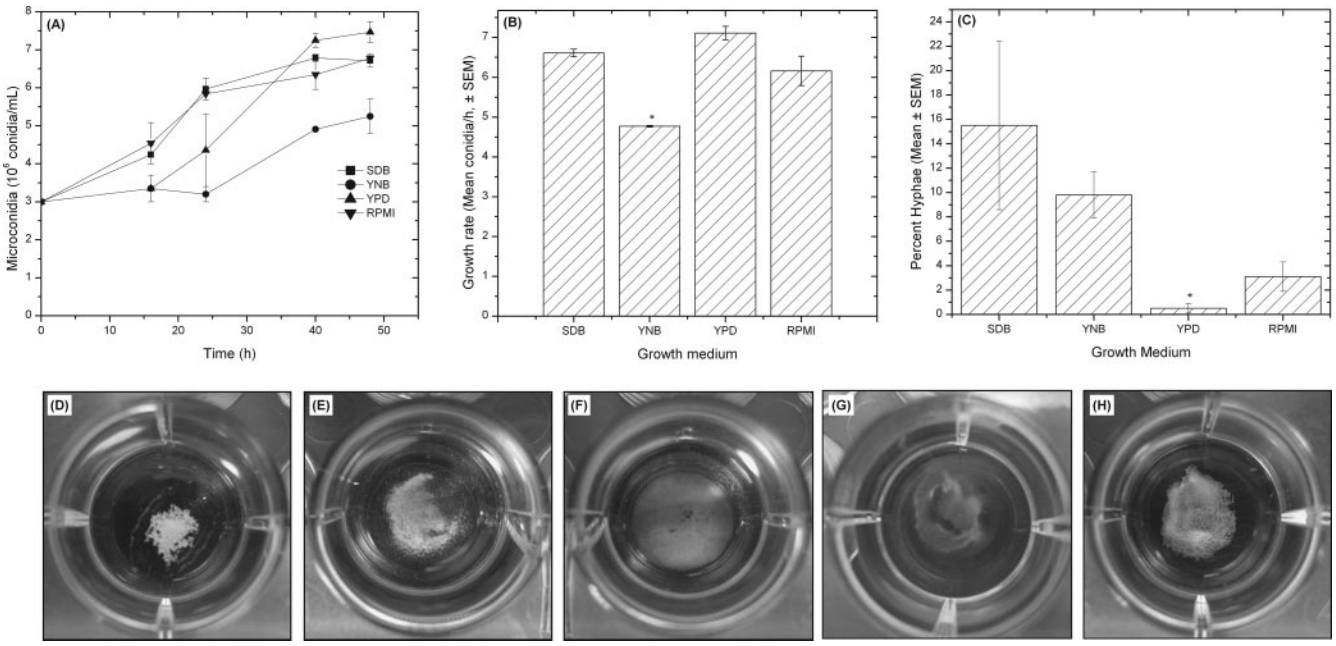

FIG. 1. Effects of different culture media on *Fusarium* growth (A to C) and gross morphology (D to H). Growth curve (A) and growth rate (B) of FSSC 1-b strain MRL8609 in different culture media are shown. (C) Number of hyphal elements present in *Fusarium* culture after 48 h of growth, expressed as a percentage of the total number of fungal conidia and hyphae {percent hyphae = [number of hyphae/(number of conidia + number of hyphae)] × 100}. (D to H) Biofilms were formed by FSSC 1-b isolate MRL8609 on soft contact lenses, and their gross morphologies were imaged using a digital camera. All lenses tested supported biofilm formation by strain MRL8609. Etafilcon A (D), galyfilcon A (E), lotrafilcon A (F), balafilcon A (G), and alphafilcon A (H) are shown.

vert100 M microscope (Carl Zeiss, Inc.). All observations were conducted with a water immersion C-apochromat objective (403; numerical aperture, 1.2).

Evaluation of antifungal activities of contact lens care solutions. The antifungal activities of MoistureLoc and MultiPlus lens care solutions against planktonic forms of *Fusarium* and *Candida* were evaluated using the ISO 14729 standalone contact lens disinfectant test (24) and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT)-based metabolic activity assay. Activities of the lens care solutions against fungal biofilms were determined by measuring their metabolic activities using the XTT-based assay.

(i) Evaluation of activities of lens care solutions against planktonic cells using ISO 14729 methodology. ISO 14729 guidelines are the standard used by the industry to demonstrate activities of contact lens care solutions against planktonically grown microorganisms (24). These guidelines specify that an active contact lens disinfectant must reduce the viability of fungal species by 1 log (90%) within the time recommended by the product's manufacturer. For evaluation of the antifungal activities of MoistureLoc and MultiPlus solutions against *Fusarium* strains ATCC 36031 (FSSC 2-c), MRL8609 (FSSC 1-b), and MRL8996 (FOSC 3-a), isolates were grown on potato dextrose agar plates for 10 days at 25°C. Conidia were harvested using a procedure based on the ISO standard and washed three times with Dulbecco's PBS plus 0.05% Tween 80. Conidial suspensions were adjusted to 5.0×10^7 conidia/ml using a hemacytometer. Subsequently, a 0.1-ml suspension of each *Fusarium* strain was mixed with 10 ml of each lens care solution and incubated at 25°C for 1, 2, 3, 4, and 20 h. At each time point, 1 ml of the resultant mixture was taken, diluted with Dey-Engley neutralizing broth (DEB) (Difco Laboratories), and spread on Sabouraud dextrose agar plates. After the plates were incubated for 2 days at 25°C, viable CFU were counted. Each strain was tested three independent times. A similar ISO 14729-based approach was used to assess the inhibitory activities of these lens care solutions against *C. albicans* SC5314.

(ii) Determination of metabolic activity of fungi using the XTT assay. The XTT-based assay has been used previously to monitor fungal damage (38), to quantify *Candida* and *Cryptococcus* biofilms, and also to determine antibiofilm activities of antifungal agents (11, 13, 14, 23, 27, 28, 31, 35, 42). In this study, we used this method to evaluate the abilities of contact lens care solutions to inhibit planktonic and biofilm forms of *Fusarium* and *Candida*. Briefly, planktonic cells grown in the absence or presence of lens care solutions were incubated with XTT (1 mg/ml) and menadione (1 mM; Sigma Chemical Co., St. Louis, MO) at 37°C for 5 h. Mitochondrial dehydrogenase-mediated conversion of XTT tetrazolium salt to a formazan product in live cells resulted in a colorimetric change, which was measured using a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA) at 492 nm.

To evaluate the ability of MoistureLoc or MultiPlus to inhibit contact lens-associated fungal biofilms, *Fusarium* or *Candida* biofilms formed on contact lenses were incubated with 4 ml of either solution in a 12-well plate for 4 h or 20 h, followed by incubation with 4 ml of DEB for 10 min to stop the action of these solutions (as per ISO recommendations). Cells treated with only DEB served as controls. Next, lens-associated biofilms were washed with PBS and their metabolic activity was determined as described above.

Statistical analyses. All experiments were performed in triplicate. Statistical analysis was performed using analysis of variance followed by the Bonferroni/Dunn post hoc test for comparisons of biofilms on different contact lenses and an unpaired *t* test for evaluating the activity of lens care solutions. A P value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The DNA sequence data generated in this study have been deposited in GenBank under accession numbers EU251484 to EU251492.

RESULTS

Optimization of growth conditions for *Fusarium* biofilm formation. To understand the pathogenesis and mechanisms involved in *Fusarium* biofilm formation on contact lenses, it is necessary to establish a robust in vitro model of such biofilms. As the first step in developing such a model, we monitored growth of MRL8609 FSSC 1-b in four different media (SDB, YNB, YPD, and RPMI) by determining the number of microconidia and percent hyphal elements formed in each medium. No significant difference was observed in the numbers of conidia/growth rates of MRL8609 FSSC 1-b grown in SDB, YPD, or RPMI (growth rate = 6.61 ± 0.095, 7.11 ± 0.17, or 6.15 ± 0.37 conidia/h, respectively; $P > 0.05$) (Fig. 1A and B).

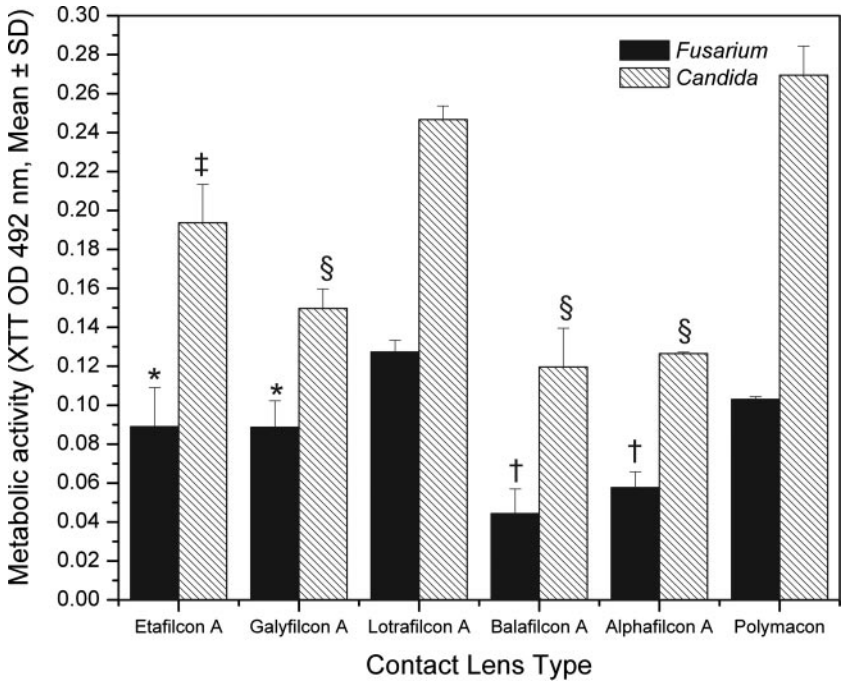

FIG. 2. Biofilm formation on soft contact lenses by *Fusarium* or *Candida* isolate. Metabolic activities of biofilms formed by FSSC 1-b isolate MRL8609 and *C. albicans* strain SC5314 on soft contact lenses were determined using the XTT assay as described in Materials and Methods. Experiments were performed in triplicate, and results are expressed as means ± SDs. The asterisk indicates a P value of <0.005 and the dagger indicates a P value of <0.0001 versus results for FSSC 1-b biofilm on a lotrafilcon A lens. The double dagger indicates a P value of <0.005 and the "§" symbol indicates P value of <0.0001 versus results for *C. albicans* biofilm on lotrafilcon A lens.

However, the growth rate of MRL8609 was significantly lower in YNB medium than in the other three media tested (growth rate = 4.77 ± 0.018 conidia/h; $P < 0.05$ for each comparison). Although no difference in the growth rates was observed between MRL8609 grown in SDB, YPD, or RPMI, the percentage of hyphae harvested from MRL8609 grown in SDB or YNB was higher than that when cultured in YPD or RPMI (Fig. 1C). It is important to note that MRL8609 growth was not influenced by enriched medium, since SDB (an enriched medium) induced abundant hyphal formation while YPD (also an enriched medium) induced only minimal hyphal formation. Moreover, YNB (a minimal medium) induced significantly more hyphal formation by MRL8609 than YPD. Since clinical presentation of *Fusarium* keratitis is commonly associated with the presence of a large number of hyphal elements in affected tissues and hyphal elements are known to assist fungal invasion of tissue (12), and since SDB supported an optimal growth rate and maximum hyphal formation, we selected it as the optimal growth medium and incubation for 48 h as the optimal time needed to form a fully mature *Fusarium* biofilm.

Gross morphologies of biofilms formed on soft contact lenses. Analysis of the gross morphologies of biofilms formed on contact lenses showed that although MRL8609 FSSC 1-b was able to form biofilms on all lenses examined (Fig. 1D to H), there was a difference in the abilities of the biofilms to adhere to the contact lenses tested. In this regard, biofilms formed by strain MRL8609 on etafilcon A and polymacon lenses were loosely attached to the surfaces of soft contact lenses and very easily dislodged when manipulated. In contrast, biofilms formed by strain MRL8609 on lotrafilcon A, balafilcon A, and alphafilcon A lenses adhered firmly to the surfaces of soft contact lenses. In general, *C. albicans* biofilms adhered firmly to the lenses, were compact, and were not easily detached, and no differences were observed in gross morphology and adhesion of biofilms formed by this pathogen on different lens types (data not shown).

Abilities of *Fusaria* and *C. albicans* to form biofilms on contact lenses vary with lens type. Since the lenses tested in this study differ in their surface properties (surface charge and water content; Table 1), we hypothesized that the ability of *Fusarium* to form biofilm will vary with different contact lenses. To test this hypothesis, we quantified biofilms formed on six commonly used lenses using the XTT-based method. Our analyses revealed that as measured by metabolic activity, strain MRL8609 FSSC 1-b formed significantly more biofilms on lotrafilcon A than on balafilcon A ($P < 0.0001$), galyfilcon A ($P = 0.0029$), etafilcon A ($P = 0.0030$), or alphafilcon A ($P < 0.0001$) lenses (Fig. 2). Additionally, significantly more biofilms were formed on polymacon lenses than on balafilcon A ($P = 0.0003$) or alphafilcon A ($P = 0.0021$) lenses. We also found that strain MRL8609 formed significantly more biofilms on both etafilcon A and galyfilcon A lenses than were formed on balafilcon A lenses ($P = 0.001$ for both comparisons). By way of contrast, the ISO 14729-recommended reference isolate ATCC 36031 FSSC 2-c failed to form biofilm on the lenses tested (data not shown). These studies revealed that among silicone hydrogel lenses, maximum biofilms were formed by strain MRL8609 FSSC 1-b on lotrafilcon A lenses while minimum biofilms were formed on balafilcon A lenses.

Since *Candida* is the second most common fungus causing

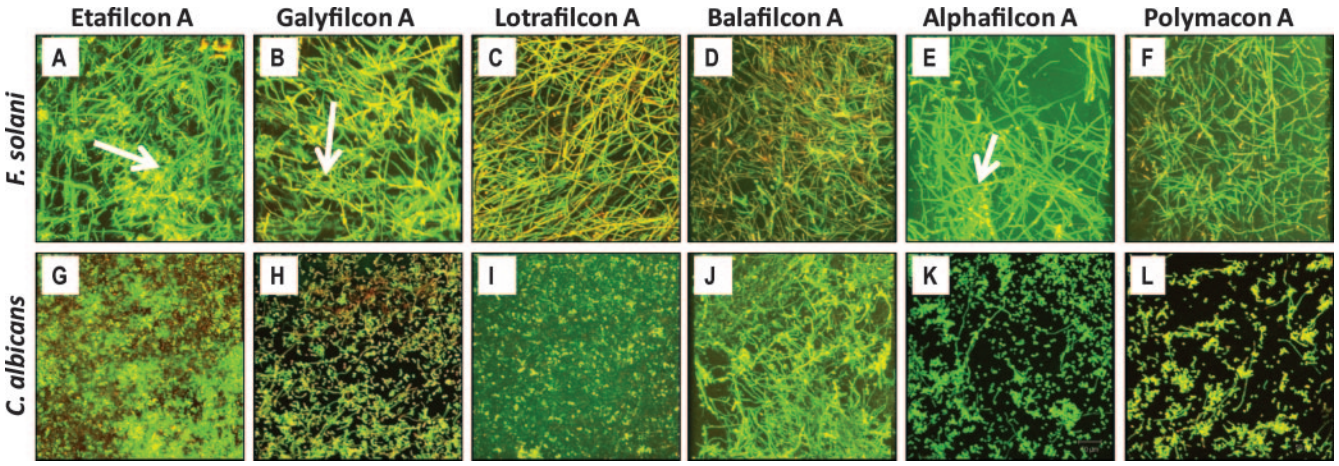

FIG. 3. Top-down architecture of *Fusarium* (A to F) and *Candida* (G to L) biofilms formed on different soft contact lenses. The FSSC 1-b strain MRL8609 or *Candida* isolate SC5314 was allowed to form mature biofilms on soft contact lenses and then was stained with ConA and FUN1 dyes. Stained lens-containing biofilms were analyzed by CSLM as described in Materials and Methods. Etafilcon A (A and G), galyfilcon A (B and H), lotrafilcon A (C and I), balafilcon A (D and J), alphafilcon A (E and K), and polymacon lenses (F and L) are shown. Arrows indicate extracellular matrix in the biofilms.

keratitis, we evaluated its ability to form biofilms on different lenses. Metabolic activity assays revealed that in similarity to *Fusarium* isolates, the *Candida* isolate tested also formed significantly more biofilms on lotrafilcon A than on galyfilcon A ($P < 0.0001$), balafilcon A ($P < 0.0001$), etafilcon A ($P = 0.0012$), or alphafilcon A ($P < 0.0001$) lenses (Fig. 2). No difference was observed in *Candida* biofilms formed on lotrafilcon A and polymacon A lenses ($P > 0.05$).

Taken together, these results demonstrate that *Fusarium* and *Candida* can form biofilms on contact lenses and that this ability is influenced by the lens type.

Lens-associated FSSC biofilms are hypha rich and have homogeneous architecture. Since the architecture of *C. albicans* biofilm is influenced by the substrate used (e.g., dentures or catheters) (15) and since contact lenses differ in their surface properties (Table 1), we hypothesized that the architecture of *Fusarium* biofilms formed on different contact lenses may also be influenced by the type of lenses used in this study. As shown in Fig. 3, CSLM analyses showed that biofilms formed by strain MRL8609 FSSC 1-b on soft contact lenses were composed of profuse hyphae. Biofilms formed on lotrafilcon A and balafilcon A were characterized by numerous filaments with yellow staining within the hyphal elements, which resulted from dual staining with carbohydrate (green; ConA) and metabolically active (red; FUN-1) dyes (Fig. 3C and D). Biofilms formed on etafilcon A, galyfilcon A, and alphafilcon A lenses contained abundant extracellular matrix (Fig. 3A, B, and E, arrows). Moreover, biofilms formed on the polymacon lens had a diffuse, granular appearance, with ConA staining visible throughout the biofilm (Fig. 3F). Additionally, biofilms formed by strain MRL8609 exhibited similar architecture and thicknesses at the center and periphery of the soft contact lenses (data not shown).

Analyses of biofilm thickness revealed that biofilms formed by strain MRL8609 FSSC 1-b on lenses with low water content were thinner than those formed on lenses with high water content. This pattern was observed for both nonionic and ionic lenses. Thus, for nonionic lenses, biofilms formed on the low-water-content lotrafilcon A and polymacon lenses (water content = 24% and 28%, respectively) were thinner than those formed on galyfilcon A and alphafilcon A, which have high water content (47% and 64%, respectively) (Fig. 4). Similarly, among ionic lens-associated biofilms, those formed on balafilcon A (with 36% water content) were thinner than biofilms formed on the etafilcon A lens (with 58% water content). These studies suggested a water content-dependent effect of the lens type on the ability of *Fusarium* to form biofilms on lenses. However, more-detailed studies are needed to determine the relevance of such an effect.

Taken together, our results clearly demonstrate that contact lens-associated *Fusarium* biofilms are hypha rich and have homogenous architecture, with some surface-dependent differences in biofilm architecture.

Architecture of *C. albicans* biofilms formed on contact lenses is heterogeneous and dependent on lens surface properties. Next, we used CSLM to determine whether the architecture of lens-associated biofilms formed by *C. albicans* is also dependent on lens surface properties and whether this architecture is similar to that of *Fusarium* biofilms. As shown in Fig. 3G to L, *C. albicans* biofilm architecture was distinctive for each lens type. For example, *C. albicans* biofilm formed on etafilcon A lenses was composed primarily of yeast cells with an abundant extracellular matrix (Fig. 3G), whereas biofilms formed on lotrafilcon A lenses consisted of sparse yeast cells interspersed within a diffuse matrix (Fig. 3I). Moreover, *C. albicans* biofilms formed on balafilcon A were rich in hyphae with few yeast cells and a distinct granular extracellular matrix (Fig. 3J). In contrast, *C. albicans* biofilms formed on galyfilcon A, alphafilcon A, and polymacon lenses were a mix of yeast and hyphae, with minimal detectable extracellular matrix (Fig. 3H, K, and L). Our analyses showed that the architecture of *C. albicans* biofilms formed at the periphery of lotrafilcon A and polymacon contact lenses differed from those formed at the center. Specifically, among biofilms formed on lotrafilcon A, the central

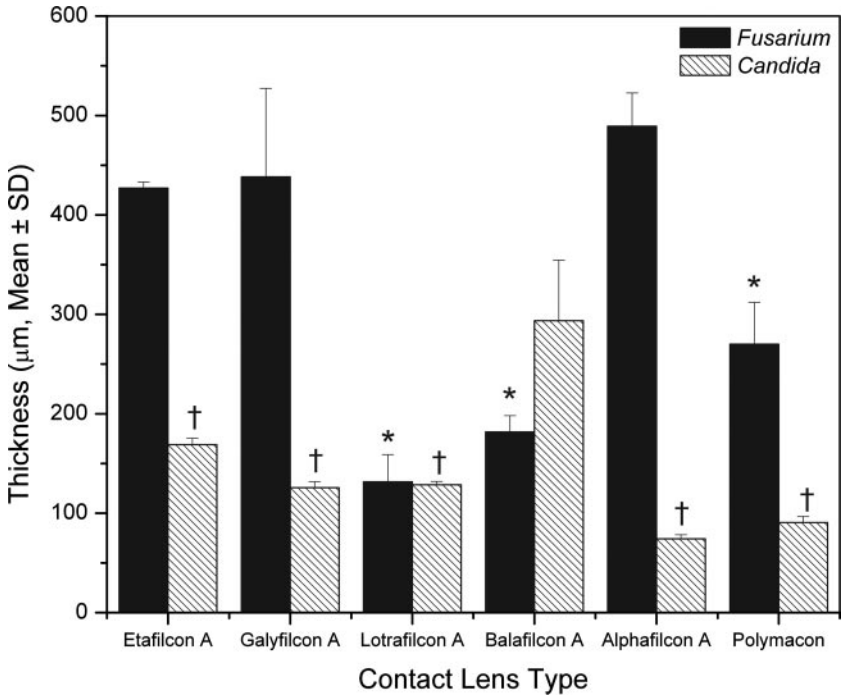

FIG. 4. Thicknesses of biofilms formed by FSSC 1-b strain MRL8609 (black bars) or *C. albicans* strain SC5314 (hatched bars) on soft contact lenses. Strains were allowed to form mature biofilms on soft contact lenses and then were stained with ConA and FUN1 dyes. The stained biofilms were analyzed by CSLM as described in Methods. Biofilm thicknesses were determined by merging all *z*-stack images into a three-dimensional projection. Side-view images were obtained using CSLM-associated software, and thickness was measured. *, $P < 0.0001$ versus results for FSSC 1-b strain MRL8609 biofilm formed on alphafilcon A lens; †, $P < 0.0001$ versus results for *C. albicans* biofilm formed on balafilcon A lens.

area contained matrix-rich, dense, "mushroom-like" structures (data not shown), similar to those seen in bacterial biofilms (9, 17, 43, 44). In *Candida* biofilms formed on a polymacon lens, the peripheral region contained sparse yeast cells, while biofilms formed in the centers of the lenses contained abundant yeast and hyphal elements. Analyses of biofilm thickness formed on lenses revealed that *C. albicans* biofilms formed on balafilcon A lenses were significantly thicker than those formed on the other lenses ($P < 0.0001$ for all comparisons) (Fig. 4). There was no significant difference in thickness between biofilms formed by *C. albicans* on the remaining five lenses ($P > 0.05$), suggesting a lack of correlation between lens water content and biofilm thickness. These results suggested that lens surface properties modulate the morphology and architecture of *Candida* biofilms but not their thickness.

Clinical isolates of *Fusarium* form biofilms on contact lenses. Having established the in vitro biofilm model, we used it to examine the abilities of three human keratitis-associated *Fusarium* isolates to form biofilms on the lotrafilcon A lens using the XTT assay. This contact lens was selected because it allowed abundant biofilm formation. As per ISO 14729 recommendations for testing activities of lens care solutions, the reaction of these disinfectants was stopped after the manufacturer-recommended contact time by adding DEB. Therefore, in our assays, we used DEB-treated cells as controls. To determine whether DEB by itself interfered with the ability of fungi to form biofilms on contact lenses, we compared the abilities of the three *Fusarium* species FSSC 1-b (MRL8609), FSSC 2-c (ATCC 36031), and FOSC 3-a (MRL8996) and *C. albicans* SC5314 to form a biofilm on lotrafilcon A lens in the absence or presence of DEB. The XTT assay revealed that exposure of cells to DEB reduced the XTT activity of the biofilm by almost 50% compared to that of the untreated control (Fig. 5). Although DEB reduced the level of biofilms formed by the isolates tested, the biofilms formed were significant and did not interfere with our ability to study the activity of contact lens care solutions against fungal biofilms. In contrast to the clinical isolates MRL8609 FSSC 1-b and MRL8996 FOSC 3-a, the ATCC 36031 reference isolate FSSC 2-c lacked the ability to form a biofilm in the presence or absence of DEB (Fig. 5).

Planktonically grown *Fusaria* and *Candida albicans* are susceptible to MoistureLoc and MultiPlus solutions. Determination of CFU after 4 h of incubation (ISO 14729 guidelines) is the standard method for evaluating activities of lens care solutions against planktonically grown microbes. Moreover, since biofilm growth is assessed by using the XTT assay and not by CFU determination and to allow comparison of the activities of lens care solutions against planktonic and biofilm-associated cells, in the current study we evaluated the effects of MoistureLoc and MultiPlus against planktonically grown fusaria and *C. albicans* using both the CFU and XTT assays. As shown in Fig. 6, planktonically grown fusaria (MRL8609 [FSSC 1-b], ATCC 36031 [FSSC 2-c], and MRL8996 [FOSC 3-a]) and *C. albicans* isolate SC5314 were susceptible to both MoistureLoc and MultiPlus solutions. Incubation of planktonically grown cells in the lens care solutions for 4 h, as suggested by the manufacturer and the ISO document, resulted in a >1 log reduction of

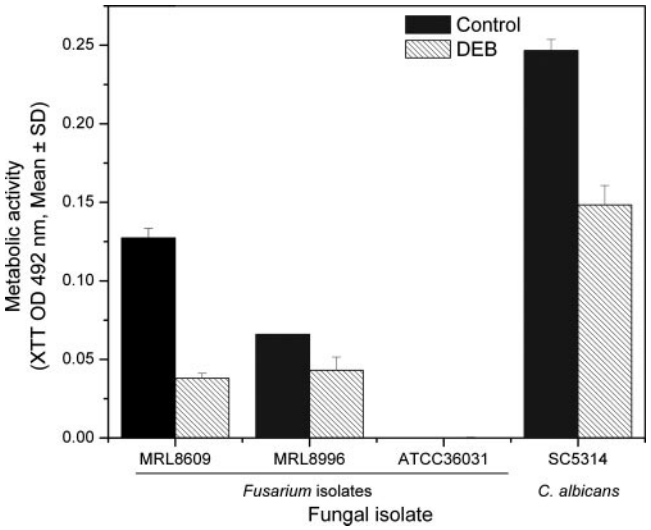

FIG. 5. Abilities of three species of *Fusarium* and *Candida albicans* to form biofilm on lotrafilcon A lens in the absence (black bars) or presence (hatched bars) of DEB. Biofilms were formed in the absence or presence of DEB on a lotrafilcon A lens as described in Materials and Methods, using the FSSC 2-c ATCC 36031 reference isolate or clinical isolate FSSC 1-b (MRL8609), FOSC 3-a (MRL8996), or *C. albicans* SC5314. Biofilms were quantified using the XTT metabolic activity assay. Data represent means (± SDs) calculated from three separate experiments.

CFU for the three fusaria isolates (Fig. 6A to C) and the *C. albicans* isolate (Fig. 6G) tested. Results from the XTT assay also revealed similar patterns (Fig. 6B, D, F, and H), with complete inhibition of metabolic activity observed after 4 h of incubation. These results demonstrated that the ISO- and XTT-based methods generate similar results regarding the activities of lens case solutions, showing that both MoistureLoc and MultiPlus are active against planktonically grown *Fusarium* and *C. albicans*.

Contact lens-associated fungal biofilms exhibit reduced susceptibility to lens care solutions. Next, we used the in vitro model we developed to test whether MoistureLoc and MultiPlus could inhibit biofilm formation by MRL8609 (FSSC 1-b), MRL8996 (FOSC 3-a), or *C. albicans* SC5314 on the lotrafilcon A lens. In contrast to the activities of lens care solutions against planktonically grown fungi (percent reductions of 53 ± 1% and 51 ± 4% compared to that for the untreated control [mean ± standard deviation [SD]) (Table 2), these solutions only partially inhibited biofilm formation following a 4-h exposure. Extending the treatment period for both solutions to 20 h resulted in slightly more inhibition of biofilms formed by MRL8906. In contrast, biofilms formed by MRL8996 were not significantly reduced by MultiPlus or MoistureLoc solutions (Table 2). However, in both cases, the lens care solutions failed to completely inhibit biofilm formation by the two phylogenetically divergent species of *Fusarium* tested. In contrast to the partial inhibitory effect of MoistureLoc and MultiPlus solutions against *Fusarium* biofilms, these agents had no inhibitory effect on biofilms formed on contact lenses at 4 h or 20 h by *C. albicans* isolate SC5314 (Table 2).

Taken together, our studies showed that biofilms formed by *Fusarium* isolates exhibited reduced susceptibility to MoistureLoc and MultiPlus lens care solutions, while *C. albicans* biofilm was completely resistant to these solutions.

DISCUSSION

In this study, we developed a reproducible in vitro model of fungal biofilm formation on contact lenses and demonstrated that *Fusarium* and *Candida*—major etiological agents of fungal keratitis—can form biofilms on contact lenses. Using the in vitro model, we showed that the amount of lens-associated biofilms formed and their composition and architecture were dependent on the species used and the type of lens tested. We also demonstrated that the XTT method can be used to evaluate fungal biofilm formation on contact lenses and to determine the inhibitory effects of lens care solutions, e.g., MoistureLoc and MultiPlus, on these biofilms. Our studies revealed that while MoistureLoc and MultiPlus lens care solutions exhibited potent activity against planktonically grown species of *Fusarium* within the FSSC and FOSC and *C. albicans*, these solutions were less effective against *Fusarium* biofilms formed on contact lenses and had no effect on *Candida* biofilms formed on contact lenses under the same conditions.

Gross morphological data showed that *Fusarium* and *Candida* differ in their ability to form biofilms on contact lenses. While the attachment of *Fusarium* biofilms varied with the lens type, *Candida* biofilms were more tightly bound to all of the contact lenses tested. The loose association of *Fusarium* mats was recently observed in studies investigating the attachment and penetration of contact lenses (4). Ahearn et al. (4) showed that *Fusarium* mats tended to be loosely associated with the lenses and could be released from the lenses by vigorous shaking or rinsing of the lens. Similar to our findings, attachment to the lens surface ranged from a loose association of conidia and hyphae to firmly attached hyphae that were difficult or impossible to remove (4). It is noteworthy that Ahearn et al. (4) reported that the two isolates of "*F. solani*" tested differed in their ability to attach to hydrogel contact lenses. Given that multilocus DNA sequence typing has shown that these isolates represent two phylogenetically distinct species within the FSSC (39) (GSU AFR4 [FSSC 1-b] and GSU 81036 [FSSC 4-a]), it is possible that some of the observed differences may be species or strain specific. Because at least 18 medically relevant phylogenetically distinct species are nested within the FSSC (51) and because human pathogenic fusaria within other species complexes are also phylgenetically diverse (39), future studies on biofilm formation employing fusaria should adopt the multilocus haplotype nomenclatural system initially presented in the 2005–2006 multistate keratitis outbreak investigation so that the findings from different studies can be compared directly. The difference in the abilities of the fusaria and *C. albicans* to tightly adhere to soft contact lenses could be due to the high propensity of *C. albicans* to adhere to surfaces (22). Another possible reason could be the ability of *Candida* to grow as yeast and hyphal forms in the biofilm while only hyphae were formed in *Fusarium* biofilms. In this regard, earlier studies showed that *C. albicans* forms a biphasic biofilm, with yeast cells forming an anchor that is strongly attached to the substrate (15). Therefore, the basal layer may account for the strong attachment of *Candida* biofilms on contact lenses.

Metabolic activity-based quantification and thickness mea-

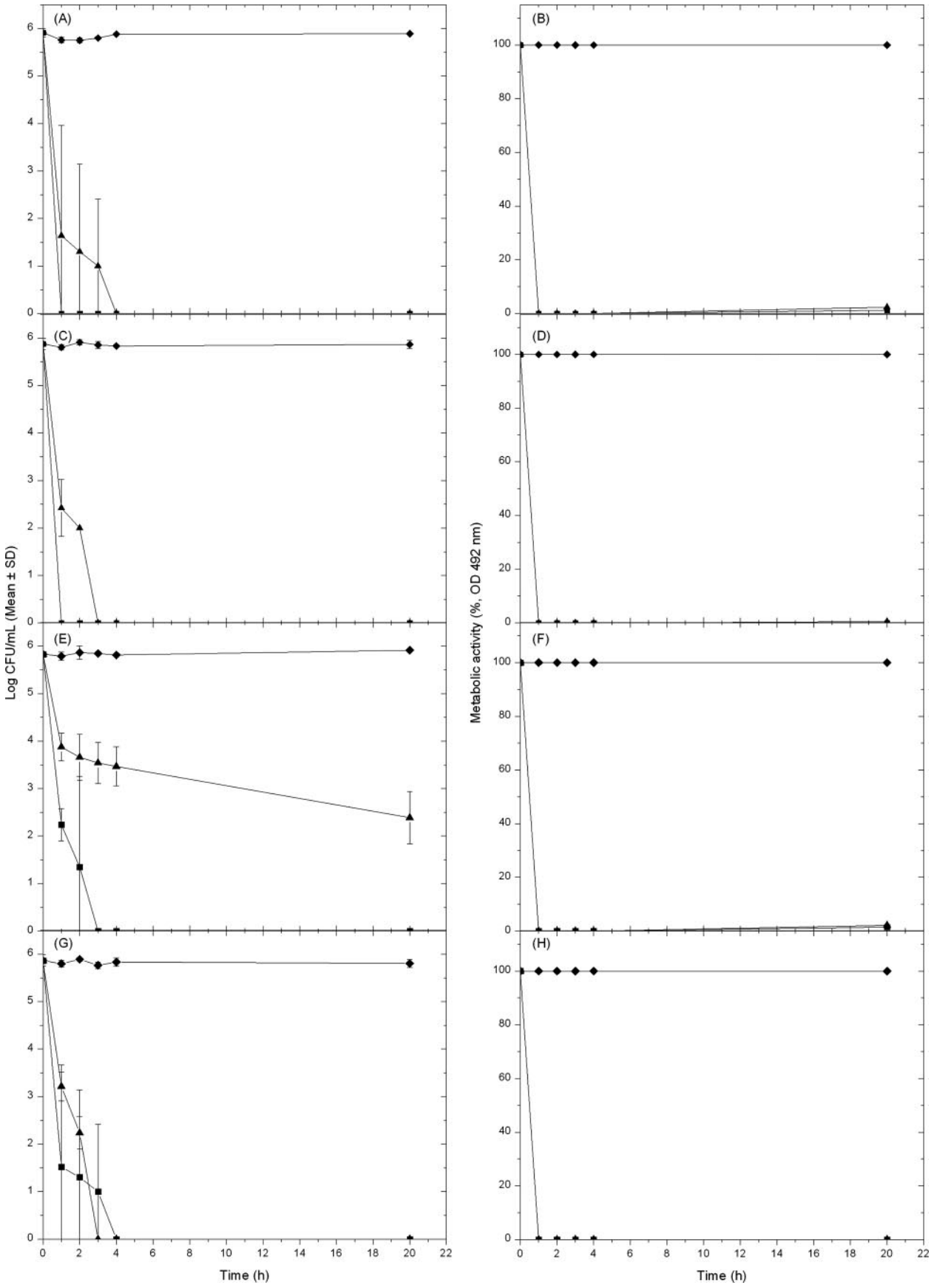
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
Log CFU/mL (Mean ± SD)
Metabolic activity (%, OD 492 nm)
Time (h)
Time (h)

TABLE 2. Effect of MoistureLoc or MultiPlus solution on metabolic activities of biofilms formed by two species of *Fusarium* or *C. albicans* on lotrafilcon A lens[a]

Strain	Lens care solution	Activity for incubation time (h) of:				*P* value[b]
		4		20		
		Mean ± SD	% Growth	Mean ± SD	% Growth	
FSSC 1-b MRL8609	None	0.0403 ± 0.0015	100.0	0.0377 ± 0.0006	100.0	0.047
	MoistureLoc	0.0190 ± 0.0010	47.1	0.0140 ± 0.0010	37.1	0.004
	MultiPlus	0.0197 ± 0.0012	48.8	0.0093 ± 0.0006	24.7	0.0001
FOSC 3-a MRL8996	None	0.0433 ± 0.0025	100.0	0.0483 ± 0.0029	100.0	0.087
	MoistureLoc	0.0230 ± 0.0046	53.1	0.0193 ± 0.0031	39.9	0.313
	MultiPlus	0.0223 ± 0.0006	51.5	0.0263 ± 0.0023	54.4	0.044
C. albicans SC5314	None	0.1483 ± 0.0124	100.0	0.1310 ± 0.0096	100.0	0.129
	MoistureLoc	0.1787 ± 0.0119	120.4	0.1337 ± 0.0159	102.1	0.017
	MultiPlus	0.1797 ± 0.0040	121.1	0.1777 ± 0.0112	135.6	0.785

[a] Biofilms were formed using an FSSC 1-b (MRL8609), FOSC 3-a (MRL8996), or *C. albicans* SC5314 isolate on lotrafilcon A lenses as described in Materials and Methods. The effect of MoistureLoc or MultiPlus lens care solution on the metabolic activities of fungal biofilms was determined using the XTT-based metabolic activity assay. Percent growth and metabolic activity (measured as optical density at 492 nm) were calculated for each lens care solution with respect to metabolic activity of biofilm grown in the absence of the disinfectant (which was considered to be 100% activity). Data represent means ± SDs for three separate experiments.

[b] *P* values were obtained for comparison of metabolic activities of biofilms formed by each isolate with incubation with lens care solution for 4 h or 20 h.

surements demonstrated that *Fusarium* and *Candida* can form biofilms on commonly used soft contact lenses and that the amount of biofilms formed by *Fusarium* was influenced by the surface properties of the lens used. In an earlier study, Chandra et al. (15) showed that modification of a polyurethane surface by adding 6% polyethylene oxide (thus modifying the surface hydrophobicity and charge) led to minimal biofilm formation by *C. albicans* on the resulting surface. A similar effect of the substrate on bacterial biofilms was shown by Okajima et al. (41). Since the lens types tested in the current study differed with respect to water content, ionic state, monomer backbone, and surface treatment, it is possible that these variables may influence the ability of fungi to form biofilms on contact lenses. Moreover, the lenses used in the current study were procured from commercial sources, and their surface properties varied in an unmatched manner. Therefore, it was not possible to unequivocally show a direct relationship between the lens ionic charge and water content. As such, additional comparative studies employing matched lens types differing in only one variable are necessary to determine the influence of surface properties on the ability of microbes to form biofilms. It is notable that the lenses used in the current study were fresh; surface properties may change in lenses that have been worn on an extended basis, and this may contribute to different levels of biofilm formation. This aspect needs to be investigated further.

Our CSLM analyses showed that while *Fusarium* biofilms were composed of a homogenous layered mesh of hyphal elements, *Candida* biofilms had a heterogeneous architecture consisting of yeast and hyphal elements. Moreover, *Fusarium* tended to form a uniform biofilm at the center and the periphery of the contact lenses, while the architecture of *Candida* biofilms formed in the center and at the periphery of the lens differed. Such differences in biofilm characteristics between organisms are not surprising and have been demonstrated earlier for different bacteria and for different candida species. For example, Kuhn et al. (31) showed that *C. albicans* isolates produced more biofilm on silicone elastomer than *Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis* isolates and that biofilms formed by *C. parapsilosis* had a patchy, "mushroom-like" structure while those formed by *C. albicans* were more continuous in nature. Furthermore, Chandra et al. (13) showed that *Candida* biofilms formed on a denture surface were composed only of yeast cells while those formed on catheter discs consisted of a basal yeast layer overlaid by one that is hyphal.

An alternative explanation for the observed differences in biofilm formation between central and peripheral regions of the lenses for *Candida* biofilms may be associated with the design of the contact lens, since all lenses used in our study had powers of +1.50 diopters and were slightly thicker in the center than the periphery. It is possible that the lenses showing differences in peripheral-to-central thickness may have an irregular surface texture which can influence biofilm formation. In this regard, Chandra et al. (13, 14) showed that *C. albicans* biofilms were thicker at raised areas present on the

FIG. 6. Effect of lens cleaning solutions against three species of *Fusarium* (A to F) or *Candida albicans* (G and H) grown planktonically. Susceptibilities of planktonically grown fungal cells were determined using the ISO 14729 methodology (A, C, E, and G) (at 0, 1, 2, 3, 4, or 20 h) or the XTT-based metabolic activity assay with an endpoint criterion of >50% inhibition compared to level for untreated controls (B, D, F, and H). The three species of *Fusarium* tested were FSSC 1-b (MRL8609) (A and B), FOSC 3-a (MRL8996) (C and D), and FSSC 2-c (ATCC 36031) (E and F), while the *C. albicans* isolate tested was SC5314. Planktonically grown *Fusarium* or *Candida* cells were either untreated (solid diamonds) or treated with MoistureLoc (solid squares) or MultiPlus (solid triangles) solutions. Percent reduction in metabolic activity was calculated for each lens care solution with respect to metabolic activity of biofilm grown in the absence of the disinfectant (which was considered to be 100% activity). Data represent means (± SDs) for three separate experiments.

surface of dentures. However, this does not explain why no differences were observed in biofilms formed by *Fusarium* at the center and peripheral areas of the lenses.

Our data did not demonstrate a direct correlation between biofilm metabolic activity, determined by the XTT assay, and its thickness, measured by CSLM. Such a lack of correlation is not surprising, since biofilms may contain fewer fungal cells embedded in a thick matrix, as reported earlier for *Candida* biofilms formed on modified surfaces (15).

Comparison of the antifungal activities of MoistureLoc against *Fusarium* and *Candida* cells grown planktonically or as a biofilm showed that planktonically grown cells were susceptible to both solutions using the manufacturer-recommended incubation time (4 h), as well as during an extended incubation (20 h). In contrast, *Fusarium* biofilms formed on contact lens were less susceptible than planktonically grown cells to MoistureLoc and MultiPlus solutions. It was not possible to completely eradicate *Fusarium* biofilms even with an extended treatment period (20 h). Interestingly, the FOSC 3-a strain MRL8996, which was obtained from a contact lens wearer with extensive corneal damage, exhibited resistance to the lens care solutions even after the extended incubation of 20 h, suggesting that some fusaria may be more resistant to these solutions than others. In addition, *Candida* biofilms were completely resistant to MoistureLoc and MultiPlus solutions. Our data are in agreement with those of May et al. (36), who examined the antimicrobial activities of a number of disinfectant solutions, recommended for use with rigid gas-permeable or hard contact lenses, against planktonic and adhered cells of bacteria and *C. albicans*. Their findings showed that while most solutions gave marked inhibition (99.99% reduction within 4 h) of planktonically growing cells, cells of all microorganisms adhering to wells of polyethylene contact lens cases showed various degrees of survival after 4, 6, and 12 h of exposure to most contact lens solutions. Similarly, Wilson et al. (49) showed that biofilms of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, and *C. albicans* formed on wells of polyethylene contact lens cases retained viability with certain soft contact lens disinfectant solutions after exposure for the manufacturer's minimum recommended disinfection times.

Lens care solutions in contact lens cases can become concentrated and often form dried films due to evaporation and because these cases are often topped off by users instead of being emptied and then refilled regularly. The effect of MoistureLoc drying on the growth of *F. solani-F. oxysporum* complex was investigated by Zhang et al. (51), who showed that MoistureLoc films on plastic surfaces of lens cases can support the growth of selective isolates of this fungus. The in vitro model developed in the current study was not suited to addressing this question. However, we found that overnight (20 h) incubation of MoistureLoc and MultiPlus solutions at 37°C did not lead to any loss of activity. Since an overnight incubation is not expected to result in major evaporation, the final concentration of active ingredients in the lens care solutions is not expected to change noticeably. Detailed investigations are needed to elucidate the role of temperature and/or compliance-related fluctuations in the concentration of lens care solutions on their antifungal activities.

Evans and Dart (21) showed that bacteria grown as biofilm show reduced susceptibility to contact lens disinfecting solutions compared to that of planktonic cells. Similarly, Kuhn et al. (32) showed that catheter-associated biofilms formed by several *Candida* species were resistant to commonly used antifungal agents. Other investigators have also shown that *Candida* biofilms were resistant to antifungal agents while the planktonic form of the same isolate was susceptible (7, 8, 13, 14, 30). It is clear that the mode of microbial growth (planktonic versus biofilm) influences the susceptibility of microbial cells to lens care solutions, where biofilms express a resistance phenotype. In contrast to our findings, Dyavaiah et al. (19) recently reported that biofilms formed by six *Fusarium* keratitis isolates on contact lenses were susceptible to MoistureLoc. The reason for the disagreement between our results and those reported by Dyavaiah et al. (19) may be related to differences in methods used to form biofilm. In our study, we allowed *Fusarium* to form biofilms in the presence of growth medium and demonstrated the presence of a carbohydrate-rich extracellular matrix (an important characteristic of biofilms) in which fungal elements were embedded. In an earlier study, Simmons et al. (46) monitored lens colonization using a method in which fungal isolates were incubated in the presence of a balanced salt solution with lens disinfectants. Dyavaiah et al. (19) followed this method to evaluate fungal biofilm formation on lenses. However, the conditions used by these investigators promote initial attachment of the fungi but do not allow biofilm formation. In agreement with the results reported by these investigators, we also found, using both the industry standard ISO 14729 and XTT-based methods, that planktonic *Fusarium* and *Candida* cells were susceptible to lens disinfectants. The recent *Fusarium* keratitis outbreaks have been associated with loss of antimicrobial activity during contact lens storage, selective growth of *Fusarium* in globules of partially dried deposits of MoistureLoc, use of a "no-rub" procedure to care for contact lenses, a general decrease in effectiveness of MoistureLoc solution compared with other lens solutions, and binding of *Fusarium* to different contact lens materials (4, 26). Our current findings suggest that the ability of *Fusarium* spp. to form biofilms on contact lenses may also contribute to their reduced susceptibility to MoistureLoc and MultiPlus.

Our results revealed that two recently isolated keratitis-causing fusaria formed robust biofilms, while the ATCC 36031 reference isolate (recommended by the ISO guidelines for evaluating the antimicrobial effects of lens disinfectants) failed to form a biofilm on soft contact lenses. The finding that the ATCC isolate did not form a biofilm is not surprising, since growth and virulence phenotypes of laboratory strains tend to change with multiple laboratory passage. Thus, it is possible that the ATCC 36031 isolate tested in this study may also have lost its virulence and hence failed to form a biofilm on soft contact lenses. Another compelling reason why the ATCC isolate recommended by the ISO should be changed is that in contrast to the two fusaria included in our experiments (i.e., FSSC 1-b [MRL8609] and FOSC 3-a [MRL8996]), which were well represented in the 2005–2006 keratitis outbreaks within the United States (16), the FSSC 2-c multilocus haplotype represented by ATCC 36031 appears to be a rare genotype in that it is represented only by a single strain from Nigeria isolated from a corneal ulcer in the mid-1970s (51). Our study, like those of others (33), revealed other inadequacies in the

testing procedures recommended by the FDA Premarket Notification [510(k)] Guidance Document for Contact Lens Care Products (48), where only one *Fusarium* isolate (24) is used to challenge the disinfectants. Currently, the disinfecting effects of contact lens care solutions for licensing purposes continue to be tested against planktonically grown microbial cells. As stated by McLaughlin-Borlace et al. (37), microbial contamination of lens storage cases is widespread, for both asymptomatic wearers and those with corneal infections, despite good compliance. Since biofilms are intimately associated with contact lenses and their carrier cases, it will be prudent to incorporate testing for activity against biofilms as part of the licensing procedures. Therefore, we propose that testing of new lens care solutions/disinfectants should include testing for activity against planktonic and biofilm-associated microbes. Moreover, a representative of the most common genotypes involved in the recent *Fusarium* keratitis outbreaks, such as FOSC 3-a, FSSC 1-a, and FSSC 2-d (16, 39), should be considered for inclusion among reference test isolates.

In conclusion, we established an in vitro model of *Candida* and *Fusarium* biofilm formation on contact lenses. The metabolic activity, thickness, and architecture of these fungal biofilms were dependent on lens type. Importantly, *Fusarium* biofilms were less susceptible than planktonically grown cells to MoistureLoc and MultiPlus solutions, which exhibited strain- and time-dependent activity against contact lens-associated *Fusarium* biofilms but were inactive against *Candida* biofilms. It is possible that the recently reported increase in the incidence of *Fusarium* keratitis among contact lens wearers may be partly due to formation of biofilms by fusaria on lens, lens cases, corneal tissue, or a combination of these surfaces. The role of biofilm formation in fungal keratitis needs to be investigated further using an expanded set of keratitis-related *Fusarium* isolates. This work is currently under way.

The in vitro model we developed for lens-associated fungal biofilms will be helpful for better understanding the biology, pathogenesis, and antifungal resistance of *Fusarium* and *Candida* biofilms and their role in contact lens-related fungal keratitis. In addition, this model has utility in evaluation of lens care solutions for their antibiofilm activity.

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